

Comparison of virulence variations on MDCK monolayers by *Escherichia coli* isolated from acute lobar nephronia and acute pyelonephritis

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SUMMARY

Escherichia coli is the most common pathogen associated with acute lobar nephronia (ALN), a clinically more severe parenchymal inflammatory disease that requires a longer duration of antibiotic treatment than acute pyelonephritis (APN). This study was conducted to unravel the virulence differences between clinical isolates of *E. coli* from pediatric ALN and APN patients. A total of 88 urinary isolates of *E. coli* were investigated. They were identified from radiologically diagnosed ALN and APN patients and had previously been molecularly characterized for important urovirulence genes. Madin-Darby canine kidney (MDCK) epithelial cells were used as an in vitro model. Multivariate logistic regression analyses indicated that ALN isolates were more likely to show adhesion ($p < 0.05$; odds ratio [OR], 3.81; 95% confidence interval [CI], 1.23-11.80) and cytotoxicity ($p < 0.001$; OR, 10.42; 95% CI, 3.03-35.89). However, no difference in the penetration ability was noted. Henceforth, the ability to adhere to and produce cytotoxicity against uroepithelial cells appears a prerequisite factor for *E. coli* to cause more severe bacterial kidney infection, such as ALN.

KEY WORDS: Acute focal bacterial nephritis, MDCK, Virulence, Bacterial adhesion, Cytotoxicity

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INTRODUCTION

With the use of renal ultrasonography and computed tomography, acute lobar nephronia (ALN), an uncommon form of renal parenchymal infections, has been diagnosed with an increasing frequency, even in the early stage (Cheng *et al.*, 2004, 2006). ALN, generally affecting one or more renal lobules, is a nonsuppurative focal form of acute

bacterial infection (Klar *et al.*, 1996; Uehling *et al.*, 2000). The clinical presentation and laboratory findings of ALN are generally similar to those with acute pyelonephritis (APN) and renal abscess (Zaontz *et al.*, 1985; Soulen *et al.*, 1989). However, patients diagnosed as ALN generally presented a longer duration of fever prior to the admission, a longer fever continuation after antibiotic treatment, and a higher C-reactive protein (CRP) value (Cheng *et al.*, 2007). Treatment regimens for the two diseases are different (Shimizu *et al.*, 2005; Cheng *et al.*, 2006). Being a more severe parenchymal inflammatory disease, ALN requires a longer three-week antibiotic treatment, rather than two-week therapy commonly used for APN (Cheng *et al.*, 2006).

E. coli is the most common causative agent for ALN (Cheng *et al.* 2004, 2006), with a frequency

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even higher than that found in the first-time urinary tract infections (UTIs) (Hoberman *et al.*, 1999). Although ALN is a more severe disease than APN, screening of the established urovirulence genes among *E. coli* isolates recovered from the two disease groups identified only one significant association between a *papG* II gene and ALN (Cheng *et al.* 2007). Furthermore, no specific genetic lineage of *E. coli* was associated with either of the two disease entities (Cheng *et al.* 2007).

To further explore the virulence factors contributing the pathogenesis of ALN, the present study examined the interactions between *E. coli* isolates and uroepithelial cells with the use of monolayers formed by Madin-Darby canine kidney (MDCK) epithelial cells. MDCK cells have been shown to be a good *in vitro* model for bacteria virulence screening (Hirakata *et al.*, 2000) or pathogenesis determination of various bacterial infections, including the uropathogenic *Escherichia coli* (Altman *et al.*, 2001; Wu *et al.*, 2001; Wade *et al.*, 2004, Lin *et al.*, 2006). Hence, the model was adopted in the present study to study the interactions, including adhesion, penetration and cytotoxicity, between a batch of well-characterized uropathogenic *E. coli* isolates and uroepithelial cells. Statistical methods were used to analyze the associations of these virulence characteristics with the presence of urovirulence genes characterized previously (Cheng *et al.*, 2007) as well as the disease entities.

MATERIALS AND METHODS

Bacteria

A total of 88 clinical isolates of *E. coli* previously collected from 46 ALN and 42 APN patients during January 2004 and October 2005 were investigated (Cheng *et al.*, 2004, 2006, 2007). The study protocol was approved by our institutional review board. Informed consent was obtained from the patients' parents after a full explanation. The inclusion criteria for the ALN and APN patients were described briefly. All patients suspected of having a first UTI, because of the presence of pyuria (>5 white blood cells/high-power field) and fever with symptoms and signs related to UTI (e.g., knocking pain, dysuria and frequency) or without focus, underwent renal ultrasonography during the first or second day af-

ter admission to the hospital. Computed tomography (CT) assessment followed immediately when the initial ultrasonographic findings met either of two criteria: evidence of unilateral or bilateral nephromegaly or a focal renal mass. For children who presented with borderline nephromegaly ultrasonographically, CT was performed when the patient remained febrile for 72 h after the beginning of antibiotic therapy. The ALN diagnosis was made on the basis of positive CT findings. APN was diagnosed clinically with no nephromegaly or based on nephromegaly with no ALN lesion on CT examination (Cheng *et al.*, 2004, 2006, 2007). All patients underwent urine cultures, and all isolates were identified using standard bacterial culture methods. A minimum of 1×10^5 colony-forming units of pathogen per milliliter of a freshly voided midstream urine, or any bacteria isolated from the urine of suprapubic aspirations, were considered positive. Only patients with *E. coli* as the sole positive isolate were included for this study. Single colonies of the *E. coli* were randomly selected from the initial culture plate and stored in 20% glycerol at -70°C until used (Cheng *et al.*, 2007).

Details for determination of ten urovirulent genes (*papG* I, *papG* II, *papG* III, *fimH*, *sfa*, *foc*, *afa*, *iutA*, *hlyA*, and *cnf1*) of these 88 clinical isolates were described in a previous publication (Cheng *et al.*, 2007).

Monolayer penetration assay

The techniques used for penetration assays were adopted from those previously described (Hirakata *et al.*, 2000; Lin *et al.*, 2006). MDCK cells maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum and 1% gentamicin were seeded at 1.5×10^5 cells/well in Transwell filter units (Costar, USA, 0.33-cm² porous filter membranes, 3.0- μ m pores). The monolayer was grown in the antibiotic-free medium with 10% fetal bovine serum at 37°C in 5% CO₂ for 4 days. The monolayers were then infected with 5- μ L of fresh grown log-phase bacteria in shaking that had been previously cultured in Luria broth (LB) in static overnight at 37°C. The multiplicity of infection was approximately 100:1 (bacteria:cell). The bacteria collected from the basolateral medium after infection at 37°C for 6 hours in 5% CO₂ were counted with appropriate dilutions on LB plates.

Salmonella enterica serotype Typhimurium SL1344 and noninvasive rabbit enterotoxigenic *E. coli* strain RDEC-1 was used as a positive control and negative control, respectively for the penetration assay. Moreover, noninvasive rabbit enterotoxigenic *E. coli* strain RDEC-1 was used as a negative control to examine the MDCK monolayer integrity. Each assay was performed in triplicate.

Bacterial adhesion assay

MDCK cells were seeded onto the round cover glass in each well of the 24-well tissue culture plate, and infected with the tested bacteria in a same manner as described in the “monolayer penetration assay” above. Six hours after infection, the bacteria supernatant was withdrawn and the non-adherent bacteria were removed by gentle washing with phosphate-buffered saline for four times. Care was taken to ensure no planktonic growth after six hours infection duration. The bacteria-adhered cover glasses were then stained by Liu’s staining. The number of adhered bacteria per cell was counted with optical microscope under 400X magnification. At least fifty MDCK cells were examined per strain and each strain was performed in triplicate.

Cytotoxicity assay

The MDCK monolayers were grown in the 24-well tissue culture plate and infected with bacteria using the same way as described in the “monolayer penetration assay” above. The supernatant was then withdrawn and centrifuged at 15000x g to remove the bacteria. The concentration of lactate dehydrogenase (LDH) released from the MDCK cells into the medium was then determined by a dry chemistry method (Johnson & Johnson, Rochester, NY, USA) performed on an autoanalyzer, KODAK EKTACHEM 250 (Lin *et al.*, 2006). Non-infected MDCK cells and bacteria without contacting MDCK monolayers were used as negative controls, whereas cells lysed with sterile deionized water was used a positive control (Lalonde *et al.*, 2000). Each assay was performed in triplicate.

Statistical analysis

All statistical analyses were performed using SAS systems for Windows (Version 8.01). Statistical comparisons of continuous data between the dif-

ferent groups were performed by Student’s *t*-test or Mann-Whitney *U*-test, where appropriate. For comparisons of nominal data, a χ^2 analysis or 2-sided Fisher’s exact test was performed, as appropriate. Multiple logistic regression methods were used to determine the virulence factors associated with ALN. Difference was considered statistically significant at $p < 0.05$.

RESULTS

The detailed clinical information for these 88 ALN and APN patients was described in our previous study (Cheng *et al.*, 2007). Results of the three *in vitro* virulence assays are shown in Figure 1. ALN isolates showed a higher MDCK-adhesion capability than APN ones (2.2 ± 6.7 bacteria/cell vs. 0.6 ± 2.0 bacteria/cell; $p = 0.001$, Mann-Whitney *U*-test). In addition, ALN isolates demonstrated a significantly higher cytotoxicity as indicated by the higher LDH levels (28.1 ± 26.7 U/L vs 9.9 ± 7.2 U/L; $p < 0.001$, Mann-Whitney *U*-test). Although the average number of bacteria that penetrated through the MDCK monolayer were higher in the ALN group, the difference was not significant ($7.4 \times 10^7 \pm 2.6 \times 10^8$ cfu/mL vs $6.8 \times 10^6 \pm 4.2 \times 10^7$ cfu/mL; $p > 0.05$, Mann-Whitney *U*-test).

If only the number of adhesion-, cytotoxicity- or penetration-positive isolates was compared, the proportion of adhesion- and cytotoxicity-positive (i.e. LDH ≥ 20 U/L as compared to the negative controls) isolates was significantly greater in the ALN group, whereas the proportion of penetration-positive isolates in these two groups was not significantly different (Table 1). Even if only the isolates showing “high” penetration capability (i.e. $> 10^5$ cfu/mL in the basolateral medium) were considered, there was still no significant difference between ALN (15/46) and APN (7/42).

The correlation between different virulence factors was further analyzed (Figure 2). Significant correlation was noted between the adhesive and penetration results: 34 of the 49 adhesive isolates as compared to 12 of the 39 non-adhesive isolates were penetrative ($p < 0.001$; OR, 5.10; 95% CI, 2.05-12.69). Adhesion results were also significantly correlated with cytotoxic (i.e. LDH ≥ 20 U/L as compared to the negative controls) data: 25 of the 49 adhesive isolates as compared to 6 of the 39 non-adhesive isolates were cytotoxic

($p=0.001$; OR, 5.73; 95% CI, 2.04-16.12). But no statistical significant correlation was noted between the cytotoxic data and penetration results ($p>0.05$).

However, if only isolates showing high penetrative capability (i.e. $>10^5$ cfu/mL in the basolateral medium) were considered (Figure 2), a significant correlation was found between cytotoxicity

and “high penetration” results: 16 of the 31 cytotoxic isolates compared to 6 of the 57 non-cytotoxic isolates were “high penetrators” ($p<0.001$; OR, 9.07; 95% CI, 3.02-27.26). Similar significance was also found between adhesion and “high penetration” results: 21 of the 49 adhesive isolates compared to 1 of the 39 non-adhesive isolates were “high penetrators” ($p<0.001$; OR, 28.50;

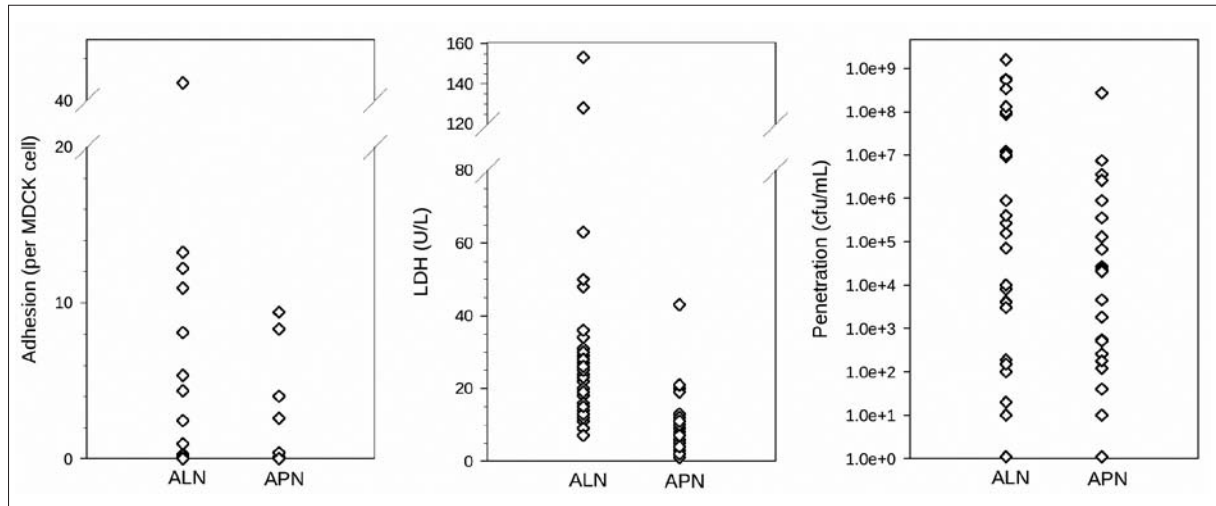


FIGURE 1 - Results of adhesion, cytotoxicity and penetration assays among the *E. coli* isolates from 46 ALN and 42 APN patients.

TABLE 1 - Virulence assessments (adhesion, cytotoxicity and penetration assays and *papG II* determination) of the 88 *E. coli* clinical isolates from patients with acute lobar nephronia (ALN) and acute pyelonephritis (APN).

Assay	No. of isolates		P	Odds ratio	95% confidence interval
	ALN	APN			
Total	46	42			
<i>PapG II</i> (+)	44	31	<0.005	7.81	1.62-37.72
<i>PapG II</i> (-)	2	11			
Adhesion (+)	34	15	<0.001	5.10	2.05-12.69
<i>PapG II</i> (+)	32	9	<0.01	10.67	1.83-62.18
<i>PapG II</i> (-)	2	6			
Cytotoxicity (+) ^a	27	4	<0.001	13.50	4.13-44.19
<i>PapG II</i> (+)	25	4	NS		
<i>PapG II</i> (-)	2	0			
Penetration (+)	26	20	NS		
<i>PapG II</i> (+)	25	15	NS		
<i>PapG II</i> (-)	1	5			

^aLactate dehydrogenase (LDH) ≥ 20 U/L as compared to the negative controls. NS: non-significant.

95% CI, 3.62-224.65). For the only one non-adhesive, high-penetrative isolate, which was also cytotoxic, most of the MDCK cells were detached from the culture plate at the end of the incubation,

and thus the counting of adhesion numbers was probably underestimated. According to the results of adhesion, cytotoxicity, and penetration assays, these ALN and APN isolates were categorized

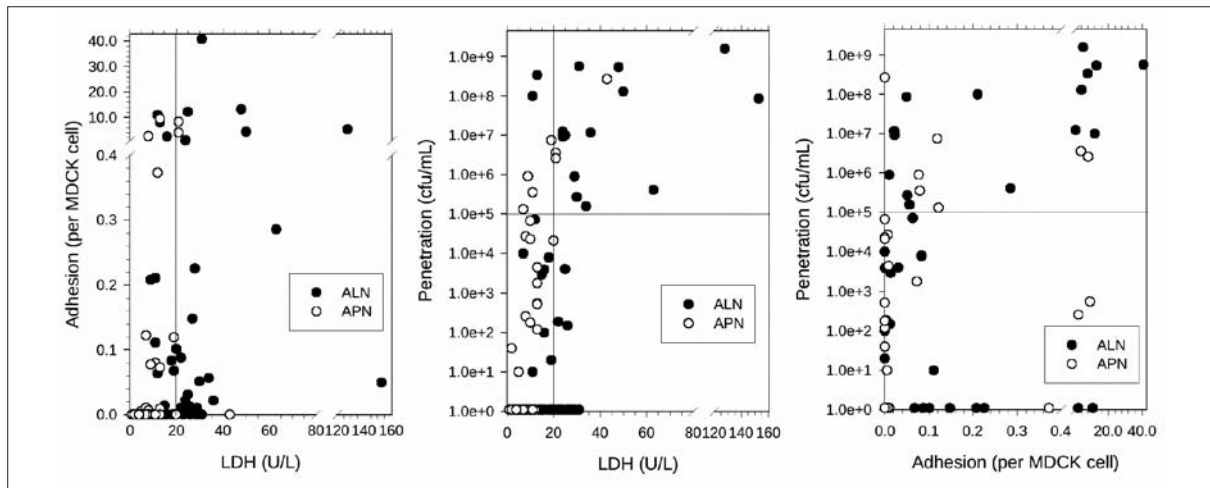


FIGURE 2 - Correlations between the adhesion, cytotoxicity and penetration assays among the *E. coli* isolates recovered from 88 patients with ALN and APN. Cytotoxic: LDH ≥ 20 U/L as compared to the negative controls. High penetrator: $>10^5$ cfu/mL in the basolateral medium.

TABLE 2 - Grouping of the 88 *E. coli* clinical isolates from patients with acute lobar nephronia (ALN, $n=46$) and acute pyelonephritis (APN, $n=42$) according to their virulence (adhesion, cytotoxicity, and penetration) properties.

Group	Adhesion ^a	Cytotoxicity ^b	Penetration ^c	No. of isolates	
				ALN	APN
A	+	+	+	16	2
A1	+	+	$>10^5$	13	2
A2	+	+	$<10^5$	3	0
B	+	+(all <40)	-	7	0
C	+	-	+	6	10
C1	+	-	$>10^5$	2	4
C2	+	-	$<10^5$	4	6
D	+	-	-	5	3
E	-	+	+	0	2
E1	- ^d	+	$>10^5$	0	1
E2	-	+	$<10^5$	0	1
F	-	+(all < 40)	-	4	0
G	-	-	+(all $<10^5$)	4	6
H	-	-	-	4	19

^a+, any MDCK cell with adhering bacteria. ^b+, lactate dehydrogenase (LDH) ≥ 20 U/L as compared to the negative controls. ^c+, any bacteria recovered in the penetration assay; sub-grouped by $>10^5$ ("high-penetrator") or $<10^5$ cfu/mL. ^dMDCK cells were detached from the culture well.

rized into 8 groups (Groups A-H) for comparison (Table 2). The *E. coli* isolates that were adhesive, cytotoxic and penetrative to the MDCK monolayer (i.e. Group A) were more likely to be isolated from the ALN than APN ($p < 0.001$; OR, 10.67; 95% CI, 2.28-49.97). Among the 49 adhesive isolates (Groups A-D), ALN isolates (23/34) were more likely than APN isolates (2/15) to be cytotoxic (Groups A and B; $p < 0.001$; OR, 13.59; 95% CI, 2.60-70.98). The significance remained even if isolates in Group B (negative for penetration) were analyzed alone ($p < 0.05$). However, it was also noted that the levels of adhesion and cytotoxicity were both low among these penetration-negative Group B isolates (all < 1 adhering bacterium/MDCK cell and LDH < 40 U/L). In contrast, among the 31 cytotoxic isolates (Groups A, B, E, and F), although the majority of the adhesive isolates were from the ALN group (23/27), the proportion of adhesive isolates among the cytotoxicity-positive population was not significantly higher in the ALN group than in the APN (23/27 vs 2/4, $p > 0.05$).

Similarly, among the 46 penetration-positive isolates (Groups A, C, E, and G), the ALN isolates (16/26) were more likely to be cytotoxic than APN ones (4/20) (Groups A and E, $p < 0.01$; OR, 6.4; 95% CI, 1.66-24.71). Furthermore, for the 42 non-penetrative isolates (Groups B, D, F, and H), the proportion of cytotoxic isolates remained significantly higher in the ALN group (Groups B and F, 11/20 vs. 0/22, $p < 0.001$). By contrast, among the 31 cytotoxic isolates (Groups A, B, E, and F), the proportion of penetration-positive or non-penetrative isolates in the ALN group remained statistically similar to that in the APN group (penetration-positive: 16/27 vs. 4/4, $p > 0.05$). In addition, among the 42 non-penetrative isolates, the ALN isolates (12/20) were more likely than APN isolates (3/22) to be adhesive to the MDCK monolayer (Groups B and D; $p < 0.01$; OR, 9.5; 95% CI, 2.10-43.05).

Further multivariate logistic regression analyses for these 88 clinical isolates revealed that ALN isolates were significantly associated with "positive adhesion" ($p < 0.05$; OR, 3.81; 95% CI, 1.23-11.80) and "positive cytotoxicity" ($p < 0.001$; OR, 10.42; 95% CI, 3.03-35.89).

PapG II was previously identified to be significantly associated with ALN infections among the 10 urovirulent genes examined (Cheng *et al.*,

2007). When this factor was compared with those from the three virulence assays, the only significant correlation was found between the existence of the *papG* II gene and the "adhesion-positive" ALN isolates ($p < 0.01$; Table 1). As for the remaining 9 urovirulent genes (*papG* I, *papG* III, *fimH*, *sfa*, *foc*, *afa*, *iutA*, *hlyA*, and *cnf1*) examined, no significant correlation was noted between the urovirulent gene and ALN or APN isolates that were adhesive, cytotoxic or penetrative to the MDCK monolayer.

DISCUSSION

The ability of bacteria to adhere to, invade, and damage the epithelial cells is important in the process of pathogenesis (Nucleo *et al.*, 2010). Adhesion of uropathogenic *E. coli* (UPEC) onto the epithelial cells has been recognized as the first event leading to various ascending UTIs, ranging from asymptomatic bacteriuria, cystitis, APN to the more severe ALN. Following that, host tissue begins to respond by initiating various defense pathways. These host defense responses, influenced by various host factors and bacterial urovirulence genes, will lead to different clinical severity in the whole UTI spectrum (Wang *et al.*, 2002; Johnson *et al.*, 2003, 2005; Cheng *et al.*, 2007; Merçon *et al.*, 2010). In the present study, *E. coli* isolates from the clinically more severe ALN patients were more capable of adhering to the MDCK cells. The phenomenon is in line with previous findings in the bacterial kidney infection model: bacteria adhesion to the uroepithelial cells by fimbrial or nonfimbrial adhesins represents an important factor in the subsequent development of upper UTI via the ascending route (Tseng *et al.*, 2001).

On the other hand, cytotoxicity was also found to play an important role in the development of ALN because both the LDH levels and the number of cytotoxic isolates were significantly higher in the ALN group. Through the higher cytotoxicity, the ALN isolates may then be able to damage the epithelial cells or induce more serious host responses as found clinically. Although genetic factors governing the cytotoxicity in these organisms remain to be explored, previous studies have shown that *E. coli* organisms are able to produce and inject toxins, such as α -hemolysin

(HlyA), cytotoxic necrotizing factor 1 (CNF1), and various autotransporter toxins, into the host uroepithelial cells, leading to different cell defense and cell damage events (Johnson *et al.*, 1991, 2005). Among the three virulence assays studied, penetration was the only factor that was not significantly associated with either disease category. However, if only the correlation among the three factors was analyzed, "high penetration" ($>10^5$ cfu/mL) was found to be significantly associated with positive cytotoxicity and adhesion, respectively (Fig. 2). This phenomenon may suggest that adhesion and cytotoxicity are factors that facilitated the penetration through the MDCK cell monolayers. The recovery of large amounts of bacteria in the basolateral medium during the *in vitro* penetration assay may just reflect the fact that the tight junction of MDCK cell monolayers could be damaged by adhesion and/or cytotoxicity, and thus bacteria were able to pass, rather than penetrate, through the cell monolayers. Similar findings have been previously reported in *Streptococcus suis* serotype 2 and its interactions with human brain microvascular endothelial cells (BMEC) (Charland *et al.*, 2000).

Instead of direct cellular invasion, *S. suis* strains adhere to and injure BMEC by producing suilysin (*S. suis* hemolysin), leading to the subsequent process of proceeding from the circulation to the central nervous system. However, a small proportion of the isolates tested in the present study (11.4%; Group G in Table 2) were still found to be penetrative without concurrent adhesion and/or cytotoxicity. These isolates may possess some specific machinery that promotes their direct penetration through the MDCK cell monolayer. A similar situation was previously reported in *Pseudomonas aeruginosa* (Hirakata *et al.*, 2000) and *Salmonella* (Finlay *et al.*, 1989). With a similar MDCK cell model, the invasive isolates were found to penetrate through, without severe damage to, the epithelial cells (Finlay *et al.*, 1989; Hirakata *et al.*, 2000). Despite that, the penetration does not seem to have a significant role in the pathogenesis of ALN as compared to APN. This conclusion was also supported by the results of the multivariate analysis which revealed only two independent factors, adhesion and cytotoxicity, significantly associated with the ALN isolates.

Nevertheless, which of the two factors, adhesion and cytotoxicity, is more important to ALN infec-

tions? It was found in the present study that among the adhesive isolates, the proportion of cytotoxic isolates was significantly higher in the ALN group than in the APN one. Even among the non-adhesive isolates, there was a borderline significance between positive cytotoxicity and ALN isolates (Groups E and F in Table 2; $p \approx 0.06$). In contrast, there was no significant difference in the proportion of positive adhesion among cytotoxic isolates from the ALN or APN group. While the ability to adhere to uroepithelial cells is important for the development of ALN, the ability to further express cytotoxicity, leading to probably the subsequent penetration through epithelial cells, apparently plays a determining role in the pathogenesis of more severe ALN, rather than just APN, by *E. coli*. This is further supported by the observation of a higher proportion of cytotoxic isolates from the ALN group than APN among the penetrative and non-penetrative *E. coli* isolates.

While multiple virulence factors/capabilities were found to be apparently associated with the pathogenesis of ALN, 12 non-adhesive isolates (Groups F-H) in the ALN group do not seem to express or only express a low level of cytotoxicity (<40 U/L) or penetration ($<10^5$ cfu/mL). Other virulence mechanisms or host factors may have involved and resulted in this severe bacterial parenchymal infection, ALN. Similar issues have also been addressed in previous studies, in which less virulent strains can cause severe upper UTIs in the hosts with predisposing factors, such as immunosuppression, urinary tract abnormalities, etc. (Tseng *et al.*, 2001, 2002; Wang *et al.*, 2002).

Our earlier study showed that a *papG* II adhesin was significantly more prevalent in pediatric patients with ALN, implying that the *papG* II allele might play a relatively more important role than the other adhesins examined (*papG* I, *papG* III, *fimH*, *sfa*, *foc*, and *afa* genes) in the development of severe renal infections (Cheng *et al.*, 2007). In the present study, the significantly higher presence of the *papG* II gene in the ALN group than in the APN group was only found in the isolates with a positive adhesion result, but not in those with a positive cytotoxicity or penetration, indicating that the adhesin, *papG* II, indeed is a paramount factor that leads to initial bacterial adhesion events, that eventually turn into more severe clinical responses observed in ALN patients.

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